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REVIEW ARTICLE

REVIEW ON COMET ASSAY: A RELIABLE TOOL FOR ASSESSING DNA DAMAGE IN ANIMAL MODELS

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ABSTRACT: The Comet assay has found worldwide acceptance for detecting DNA damage and repair in prokaryotic and eukaryotic cells. The variability in the results of the Comet assay is largely due to its sensitivity and minor differences in the conditions of various laboratories as well as the effect of confounding factors in human studies (lifestyle, age, diet, inter individual, and seasonal variation). The limitation of the Comet assay is that it only detects DNA damage in the form of strand breaks. It is therefore required that the in vitro and in vivo testing is conducted according to the Comet assay guidelines and appropriately designed multi-laboratory international validation studies be carried out. Guidelines for the in vitro as well as in vivo Comet assay have been formulated. Recently, issues relating to study design and data analysis in Comet assay were discussed by the International Workgroup on Genotoxicity Testing where particular attention was given to the alkaline version (pH>13) of the in vivo Comet assay and recommendations were made for a standardized protocol, which would be acceptable to international agencies. Consensus was also reached on the need for an international validation study to stringently evaluate the reliability and accuracy of the in vivo Comet assay (as well as in vitro versions). These recommendations are also aimed at reducing the variability arising in inter-laboratories studies. In vivo Comet assay has been accepted as the first-tier screening assay for assessment of DNA damage in rodents by the Committee on Mutagenicity, united kingdom, and international validation studies are underway supported by ECVAM, Japanese Centre for Validation of Alternative Methods (JaCVAM), United State Interagency Coordinating Committee on Validation of Alternative Methods (ICCVAM), United State National Toxicology Program Interagency Centre for Evaluation of Alternative Toxicological Methods (NICEATM), and Japanese Environmental Mutagen Society.
KEYWORDS: Comet, Assay, Tool, Damage, DNA, Animal.

INTRODUCTION

There are many methods available for the scoring of DNA damage and repair. The most commonly used are the bacterial Ames test, the scoring of chromosome aberrations, micronuclei and sister chromatid exchanges (SCE) in proliferating cell populations and, for DNA repair studies, the detection of DNA repair synthesis with the unscheduled DNA synthesis assay. These methods are used for laboratory investigations as well as for human bio-monitoring and for investigations of environmental pollution (genotoxicology testing of environmental samples and/or studies in different species living in the particular environments). Furthermore these methods were also used to investigate the anti-carcinogenic/anti-mutagenic properties of natural products. These methods have been and remain very useful but nevertheless they have a number of important shortcomings, among them the requirement for proliferating cell populations. The fact that DNA damage that will be investigated with cytogenetic methods must be processed into microscopically visible lesions is another shortcoming, as only damage with immediate importance can be detected. The technique are sometimes laborious in view of the above shortcomings and because of the need of more rapid short term screening tests, further tests were developed in recent years and others are still in development.

Östling and Johannson (1984) developed a Micro-electrophoretic technique under neutral conditions to study DNA damage in individual cells after gamma-irradiation. The Single Cell Gel Electrophoresis assay (also known as comet assay) is an uncomplicated and sensitive

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technique for the detection of DNA damage at the level of the individual eukaryotic cell. It was first described by Singh *et al.*, (1988). It has since gained popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. It involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline (pH>13) conditions, and electrophoresis of the suspended lysed cells. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage. The concept underlying the SCGE assay is that undamaged DNA retains a highly organized association with matrix proteins in the nucleus. When damaged, this organization is disrupted. The individual strands of DNA lose their compact structure and relax, expanding out of the cavity into the agarose. When the electric field is applied the DNA, which has an overall negative charge, it is drawn towards the anode, which is positively charged. Undamaged DNA strands are too large and do not leave the cavity, whereas the smaller the fragments, the farther they are free to move in a given period of time.

Therefore, the amount of DNA that leaves the cavity is a measure of the amount of DNA damage in the cell. The image analysis measures the overall intensity of the fluorescence for the whole nucleoid and the fluorescence of the migrated DNA and compares the two signals. The stronger the signal from the migrated DNA the more damage there is present. The overall structure resembles a comet (hence "comet assay") with a circular head corresponding to the undamaged DNA that remains in the cavity and a tail of damaged DNA. The brighter and longer the tail, the higher the level of damage. However, the neutral conditions they used, allowed the detection of only DNA double strand breaks. Later, the assay was adapted under alkaline conditions by Singh *et al.*, (1988), which led to a sensitive version of the assay that could assess both double- and single-strand DNA breaks as well as the alkali labile sites expressed as 'frank strand breaks in the DNA'. Since its inception, however, the assay has been modified at various steps (lysis, electrophoresis) to make it suitable for assessing various kinds of damage in different cells (Collins, 2004). The assay is now a well-established, simple, versatile, rapid, visual, and a sensitive, extensively used tool to assess DNA damage and repair quantitatively as well as qualitatively in individual cell populations (Olive, 2002). Some other lesions of DNA damage such as DNA cross-links (e.g., thymidine dimers) and oxidative DNA damage may also be assessed using lesion-specific antibodies or specific DNA repair enzymes in the Comet assay. It has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies, genotoxicity testing, and human biomonitoring (Kassie *et al.*, 2000). Relative to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, alkaline elution, and micronucleus assay, the advantages of the Comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one break per 1010 Da of DNA) (Gedik *et al.*, 1992), requirement for small number of cells (~10,000) per sample, flexibility to use proliferating as well as non-proliferating cells, low cost, ease of application, and the short time needed to complete a study. It can be conducted on cells that are the first site of contact with mutagenic/carcinogenic substances (e.g., oral and nasal mucosal cells).

The comet assay is a versatile technique for detecting damage and with adjustments to the protocol can be used to quantify the presence of a wide variety of DNA altering lesions (damage). The damage usually detects single strand breaks and double strand breaks. It is sometimes stated that alkaline conditions and complete denaturating of the DNA is necessary to detect single strand breaks. However this is not true, both single- and double strand breaks are also detected in neutral conditions. In alkaline conditions, however, additional DNA structures are detected as DNA damage: AP sites (abasic sites missing either a pyrimidine or purine nucleotide) and sites where excision repair is taking place. The comet assay is an extremely sensitive DNA damage assay. This sensitivity needs to be handled carefully as it is also vulnerable to physical changes which can affect the reproducibility of results (Collins *et al.*, 1997b). The most common form of the assay is the alkaline version although there is as yet no definitive alkaline assay protocol. Due to its simple and in expensive setup, it can be used in conditions where more complex assays are not available.

1.1. Forms of Comet Assay

There are different forms of the comet assay in order to achieve various objectives; various modifications of the comet assay have been developed. These allow us to look at the in vitro effects and explore mechanisms of action of potentially genotoxic or genoprotective agents. The comet assay can be applied to nearly all eukaryotic cells (red blood cells cannot be used as they lack a nucleus), but lymphocytes are the most commonly used type of cell in human

studies. Under controlled conditions, cells can be incubated with the test agent in vitro, and the resulting DNA damage in the treated cells can then be measured using the comet assay. Of more interest perhaps, is the effect of a 'challenge' to DNA after the cells have been exposed to a putative protective agent. This approach is widely used in determining the potential genoprotective effects of dietary antioxidants, herbs and phytochemicals. The comet assay can also be used as an 'in vivo' biomonitoring tool for investigating the effects of foods, food components, or supplements that are believed to have a genoprotective effect. A brief description of the various modifications of comet assay procedures is given below, and summarized in Table 1.

1.2. Neutral Comet Assay

The comet assay was first introduced by Ostling and Johanson (1984), who used what is termed a 'neutral' pH of 9.5 for lysis and electrophoresis. This pH is below the limit for DNA unwinding, and was reported by Singh *et al.,* (1988) to detect only double strand breaks (DSB), with more strongly alkaline conditions (pH 10 or above) needed for unwinding and detection of single strand breaks (SSB). However, SSBs are detected in the neutral or mildly alkaline comet assay, which has been shown to have the same limit of detection of DNA damage as the alkaline comet assay (Collins, 2004), although the use of neutral pH does affect the comet image obtained. The comet tails are less pronounced at neutral, and this lessens the sensitivity, or DNA damage score gradient, of the assay (Angelis *et al.,* 1999; Collins, 2004). This can be an advantage when a less sensitive method is needed, for example when investigating cells that have a large amount of background, or induced damage is high (Angelis *et al.,* 1999).

1.3. Alkaline Comet Assay

Singh *et al.,* (1988) presented the alkaline version of the comet assay, in which DNA is allowed to unwind at pH >13. In the original version, DNA damage was measured as the migration distance of DNA from the nucleoid. Olive *et al.,* (1990) also used alkaline conditions. This group introduced the term 'comet assay' and also developed the concept of the tail 'moment', a combination of tail length and DNA content, as a measure of DNA damage. The strongly alkaline conditions make for clearer images, and a steeper gradient of response. Besides SSBs, other types of DNA damage, such as alkaline labile sites (ALS), can be detected at strongly alkaline conditions (Tice *et al.,* 1997). SSBs are formed from alkali-labile sites at pH >13, revealing otherwise hidden damage. Employing milder alkaline (pH=12.3) conditions prevents conversion of alkaline labile sites into breaks (Olive *et al.,* 1990). Therefore, by modifying the pH of the lysis/unwinding and/or electrophoresis steps over the range 9.5-13.5, comet assay models of different sensitivity (but of similar limits of detection) can be applied (Collins *et al.,* 1997a; Angelis *et al.,* 1999).

1.4. Enzyme Linked Comet Assay

The use of enzymes can produce a comet assay model of greater sensitivity and, in addition, a more specific assay can be developed. In addition to SSBs, DSBs and purinic sites, other types of damage, such as oxidized bases or UV-induced dimers, which do not cause strand breaks, exist. These types of DNA damage cannot be detected unless lesion-specific enzymes are added (at the post-lysis stage) to create breaks at the sites of damage. Enzymes that have been used to date include Endonuclease III, which detects oxidized pyrimidines (Collins *et al.,*1993), formamidopyrimidine glycosylase (FPG), which detects oxidized purines (Dušinská and Collins, 1996), uvrABC, an enzyme complex that can be used to detect UV damage on bulky lesions (Dušinská and Collins, 1996), methyladenine DNA glycosylase II (AlkA) that reveals 3-methyladenine sites (Collins *et al.,* 2001b), and uracil glycosylase (UDG), which exposes sites of misincorporated uracil (Duthie and Hawdon, 1998). Besides the use of enzymes to reveal specific lesions, the enzyme proteinase K has been used to relax highly compacted DNA such as that found in sperm (Duty *et al.,* 2002). The high DNA density in sperm hinders DNA movement during electrophoresis, and proteinase K assists relaxation and histone removal (Singh *et al.,* 1996).

1.5. Fluorescence in situ hybridization–Comet Assay (FISH-comet)

Fluorescence in situ hybridization (FISH) can be used to identify chromosomes, or to detect a particular gene, or damage to this, in the comet image. The combination of the comet assay and FISH has been described by McKelvey-Martin *et al.,* (1998). This combination provides also the opportunity to investigate domain-specific DNA repair, and can be used to locate a specific gene in the three-dimensional chromosomal structure. The different rates of repair of damage in specific genes relative to the genome overall have also been investigated by this modified form of the comet assay (Horváthová *et al.,* 2004).

1.6. Lysed Cell Comet Assay

In the original version of the comet assay, treatment of cells with the potential genoprotective agent of interest comes before the lysis step. The cell membrane prevents entry of certain types of molecule into cells owing the large molecular size or high polarity, preventing assessment of the direct effects of such agents on DNA. Nonetheless, geno protection in the original version of comet assay might be exerted indirectly. For example, exposure to some dietary components may up regulate cellular defences so that DNA becomes more resistant to damage, or DNA repair mechanisms may be enhanced, leading to apparent geno protection by the test agent. Several groups have modified the comet assay procedure by lysing embedded cells before treatment with testing agents (Szeto *et al.*, 2002). This allows direct contact of 'naked' nuclear DNA with the agent under study, and removes the possibility of cellular response or adaptation. Another modification is lysing the embedded cells by briefly immersing the gel in 1% Triton X-100 detergent. This dissolves the cell membrane but not the nuclear membrane (Collins *et al.*, 2001a). The different versions (whole cell, or 'standard', partially lysed, and 'naked DNA') of the comet assay offers a means of assessing direct versus indirect mechanisms of action of geno protective agents.

Table 1: Overview of different approaches used in the comet assay and their application in nutraceutical Research.

Version	Description/Characteristics	Advantages/Application	Reference
'Neutral' comet assay	Lysis and electrophoresis performed at pH 9.5; less DNA unwinding and less pronounced comet tails; detects single strand and double strand breaks/similar limit of detection but less sensitive than alkaline version	Useful in situations where less sensitivity is needed, e.g., when background damage or induced damage is high	Ostling & Johanson 1984; Singh, 1988; Angelis et al., 1999; Collins 2004
Alkaline comet assay	Lysis/unwinding/electrophoresis performed at more strongly alkaline conditions: at pH >13, alkaline labile sites are converted to single strand breaks; altering pH over the range 9.5-13 alters sensitivity	Clearer comet images are obtained; greater response to damage seen compared to neutral version; commonly used 'standard' comet assay model; usually adopted when investigating possible protection against damage inducers, e.g. H_2O_2	Singh, 1988; Olive et al., 1990; Angelis et al., 1999 ;Tice et al., 2000
Enzyme assisted comet assay	Specific enzymes used immediately after lysis to transform susceptible sites to single strand breaks; enzymes used include Endo III (reveals oxidized pyrimidines), FPG (reveals oxidized purines), uvrABC (reveals UV damage); assay has increased sensitivity, and enhanced specificity for particular types of DNA lesions	Can detect specific types of damage; has enhanced sensitivity; is useful for looking at differences in basal damage after supplementation	Collins et al., 1993; Dušinská & Collins, 1999
Proteinase K-assisted unwinding	Enhances unwinding of densely compacted DNA, allowing damage to be detected	Can enable DNA damage in sperm to be assessed	Duty et al., 2002
Fluorescent in situ hybridization comet assay (FISH comet)	Uses gene 'tagging' with fluorescent markers and so can be used to visualize a specific gene in the three-dimensional chromosomal structure	Enables domain-specific DNA repair to be investigated; a specific gene in the three-dimensional chromosomal structure can be located; different rates of repair of damage in specific genes relative to the genome overall can be measured	Santos et al. 1997; McKelvey-Martin et al. 1998; McKenna et al. 2003; Horváthová et al. 2004
Lysed cell or partially lysed (nucleus intact) comet assay	Lysis of cell and nuclear membranes or cell membrane only (leaving nucleus intact) prior to exposure to test agent, thus exposing 'naked' DNA or nucleus to the agent	In conjunction with the whole cell, 'standard' version, this version can be applied in assessment of direct versus indirect mechanisms of action of genoprotective agents	Kasamatsu et al., 1996; Thomas et al., 1998; Collins et al., 2001a ; Szeto et al., 2002

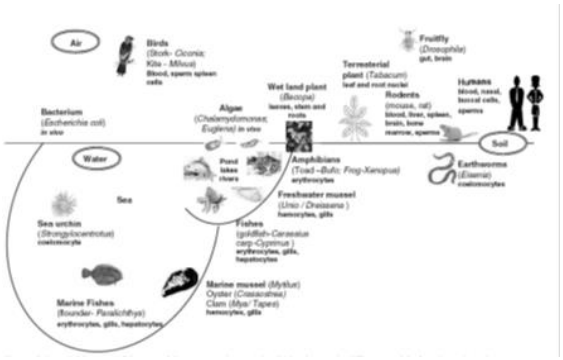

Figure 1: The Present Review deals with various models ranging from bacteria to man used in the Comet assay for assessing DNA damage.

COMET ASSAY PROTOCOL

The comet assay is a relatively simple, but sensitive and well validated tool for measuring strand breaks in DNA in single cells. Cells are embedded in a thin layer of agarose on a microscope slide and lysed with detergent and high salt solution. This procedure also removes proteins and histones, leaving a nucleoid from each embedded cell lying within a cavity in the gel. The presence of breaks in DNA causes a local relaxation in the super coiled loops of DNA in the nucleoid. When a small electrical charge is passed through the gel, the relaxed areas of the DNA loops are pulled towards the anode, forming a comet 'tail', the DNA in the nucleoid being the comet 'head'. Comets are visualized by fluorescent microscopy, and the amount of DNA in the tail, relative to the head, is proportional to the amount of strand breaks. Cells can be incubated in vitro with an agent of interest prior to the comet assay, and the resulting DNA damage can then be measured. The effect of a 'challenge' to DNA after treated cells have been exposed to a putative protective agent can also

be investigated, and cells (usually lymphocytes) can be collected pre- and post-supplementation with an agent of interest to assess possible genoprotective or genotoxic effects.

Cells is gotten either from the mouse or humans and they cells would be suspended following the normal cells procedure for preparation, after that the viability of the cell would be determined by the Trypan blue dye exclusion technique before conducting the Comet assay. Slides would be prepared according to the method of Bajpayee *et al.*, (2005). Briefly, conventional 75 3 25 mm glass slides with 19-mm frosted ends were precoated with 1% NMA, which formed the first layer or base layer. The second layer of 80ll and 1% LMA (prepared in PBS), would be applied over the first layer. Slides would be prepared in duplicate for the controls and each concentration of the sample. Cover slips (24 3 60 mm, No. 1; Blue Label Scientifics) would immediately place over the second layer, and the slides were chilled on ice for 10 min to solidify the agarose. The cover slips were removed and a third layer of 90 ll 0.5% LMA was applied, the cover slips replaced, and the agarose allowed solidifying over ice for 10 min. Finally, the cover slips were removed and the slides would be immersed in freshly prepared, chilled lysing solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), with 1% Triton X-100 added just before use]. The slides remained in lysing solution for at least 4 hr at 48C.

2.1. Electrophoresis

Electrophoresis would be carried out according to the method of Singh *et al.*, (1988). Slides would be incubated in chilled electrophoresis buffer (1 mM Na2EDTA and 300 mM NaOH, pH >13) for 20 min to allow the DNA to unwind, and electrophoresis was conducted using the same buffer for 30 min at 48C and 24 V (0.7 V/cm), adjusting the current to 300 mA. All these steps were conducted under dim light. After electrophoresis, Tris buffer (0.4 M Tris, pH 7.5) was gently added drop-wise to neutralize excess alkali; the buffer was allowed to remain on the surface of slides for 5 min. This neutralizing procedure was repeated three times.

2.2. Staining

Each slide would be stained for 5 min with 75 ll of 20 lg/ml EtBr (Ethedium Bromide), dipped into chilled distilled water to wash off excess EtBr (Ethedium Bromide) and covered with a cover slip. The slides would be placed in a dark, humidified chamber to prevent drying of the gel and analyzed within 24 hr.

2.3. Slide Scoring

Slides would be scored under blind code, using a fluorescence microscope (Leica DMLB, Wetzlar, Germany) equipped with appropriate filters (N2.1, Ex. 515–560 nm, band pass; Em. 590 nm, long pass). The final magnification was 4003. The microscope was connected to a computer through a charge coupled device camera that transported images to image-analysis software (Komet 3.1; Kinetic Imaging).

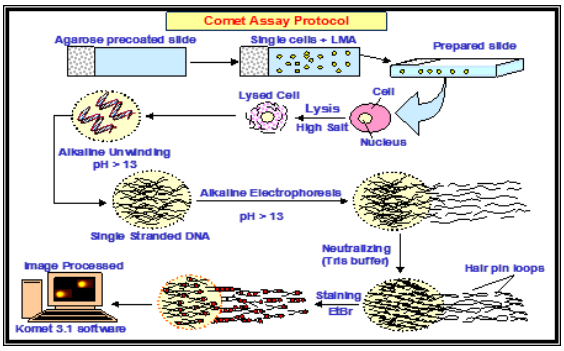

Plate 1: Showing Alkaline Comet Assay Protocol (Bajpayee *et al.*, 2005).

SCORING OF DNA DAMAGE

Scoring of DNA damage can be performed by visual (manual) scoring, using a fluorescent microscope, or by using an automated software scoring system with an image capture device attached to the microscope. In visual scoring, a grade is given to each comet image according to the intensity and length of the comet tail visualized. Illustrative examples of the different grades are given in Plate 2. There are several software packages that are custom-made to translate the captured comet image into a DNA damage index. These packages include Komet, Colour morph Comet Assay, Metasystems, Comet Analysis System, Auto Comet (http://www.cometassay.com).

Indices of damage that can be used include length of the comet tail, tail inertia, and the tail moment. However the % DNA in the comet tail has been shown to be linearly related to DNA damage (Figure 2), is widely used, and correlates well with visual scores (Figure 3). Use of tail moment (or tail inertia) is to be discouraged, as it does not have standard units, and so gives no information about the kind of comet being described, or the level of damage sustained by the cells. In contrast, a comet with, say, 60 % of DNA in the tail is easily visualised, and we can assess the amount of damage present in the cells. The common practice is to score 50 comet images per gel, and the mean DNA damage score for each gel is calculated. Generally two or three gels per treatment are scored, and the mean and SD (or SEM as appropriate) of the three gels are

presented. When using the comet assay, and when individual differences are being tested, for example between subjects given placebo and subjects given antioxidants, the statistical analysis should be based on the overall mean scores of the mean gel scores for each subject, and not on individual comet image values.

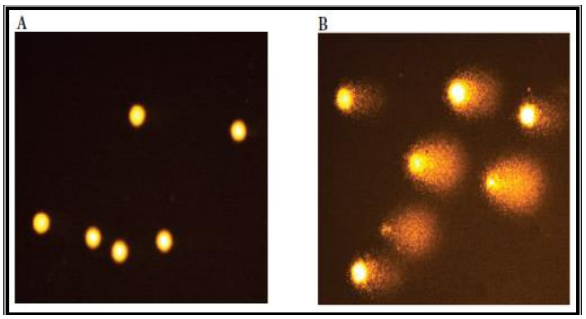

Plate 2: Representative comet images showing different levels of damage in visual scoring: (A) Score 0 (undamaged DNA); (B) various degrees of damage from minor (Grade 1) to Severe (Grade 4).

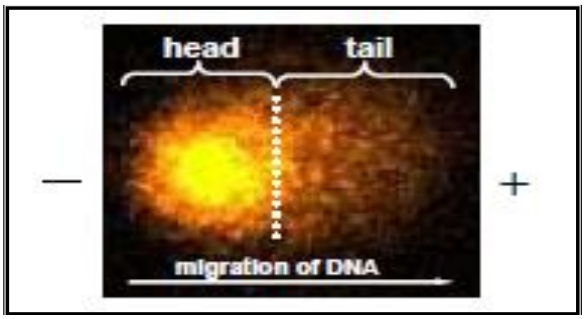

Plate 3*:* Showing the migration of DNA damage from embedded nuclei with head (Undamaged DNA) and tail (damaged DNA) (Bajpayee *et al.*, 2005).

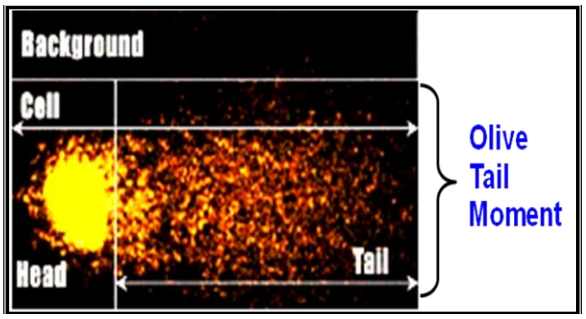

Plate 4: Showing DNA damage Measurement (Source: Bakare *et al.,* 2007). Where; Tail length (mm) - distance from the centre of the head to the end of tail Tail DNA (%) - is the fraction of DNA in the tail Olive Tail Moment-is the product of the tail length and (arbitrary units) fraction of DNA in the tail.

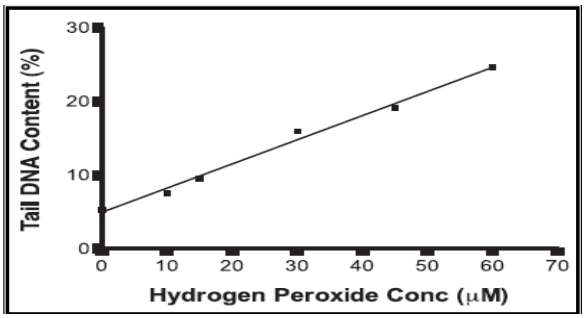

Figure 2: Showing DNA damage to lymphocytes after oxidant challenge induced by Hydrogen peroxide shows a linear response (r2= 0.9912). Results are the mean Scores of two gels per treatment, with 50 cells scored per gel.

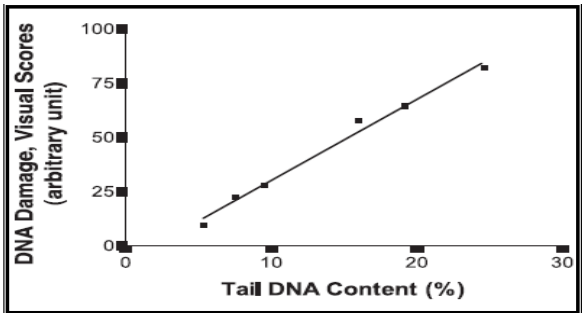

Figure 3: Showing Visual (manual) DNA damage score correlates well with the computer-generate score for %DNA in comet tail (r2= 0.9874). Results are the mean of two gels (Each with 50 cells scored per gel) per treatment.

CELL TYPES USED IN COMET ASSAYS

Theoretically, the comet assay can be applied to any eukaryotic cell, however, the most frequently used types of human cell are white cells, mainly lymphocytes, though granulocytes can also be used (Vijayalaxmi *et al.,* 1993). White cells are easily harvested from venous or capillary blood, whereas it is generally difficult to obtain other types of nucleated cells from human subjects. Beside human lymphocytes (Stylianos *et al.,* 1999) other cell types such as spermatocytes, fibroblasts, epithelial cells from the buccal (oral) cavity (Valverde *et al.,* 1997; Szeto *et al.,* 2003), stomach, tear duct, nose and bladder (Kassie *et al.,* 2002) have been used, although not always successfully. Buccal cells, for example, are highly resistant to lysis and these surface cells also have a high level of background, or basal, DNA damage (Szeto *et al.,* 2003). Malignant cells from adenocarcinoma (Stylianos *et al.,* 1999) and lymphoma also have been studied. Various white cell lines also can be used (Stylianos *et al.,* 1999), such as RAJI and TK6 from B-cell lines and HUT-78 from T-cell lines. Malignant cell lines, such as A1698 from bladder carcinoma, WiDr and HT-29 from colon carcinoma, Siha and HeLa from cervix

carcinoma, MeWo and HT-144 from melanoma, and DU-145 from prostate carcinoma also have been studied. In addition to these various human cell types, animal cells can be used. These include lymphocytes, liver, pancreas, kidney, testis and brain cells, thymocytes, splenocytes and bone marrow cells, mucosal epithelial cells and embryonic cells. Cultured animal cells lines, such as L5178Y and SCCVII from mouse and CHO and V79 from hamster, also have been studied. Plant tissue also can be applied in the comet assay (Singh *et al.,* 1996; Angelis *et al.,* 2000; Gichner *et al.,* 2004).This wide range of diverse range of cells types used shows the versatility of the technique.

ADVANTAGE OF COMET ASSAY

The advantages of the comet assay are its speed, simplicity, low cost, the small number of cells required (<10,000 cells), its sensitivity, and its widespread applicability with eukaryotic cells, whether proliferating or non-proliferating (Tice *et al.,* 1995; Collins, 2004). Furthermore, effects on different cell types can be explored. This is an advantage because genotoxic and genoprotective effects can be tissue or cell type specific (Singh *et al.,* 1988; Burdon, 1999). Another advantage of the comet assay is its flexibility; different combinations of unwinding and electrophoresis conditions and lesion-specific enzymes can be used to detect different types and levels of DNA damage. Owing to the numerous advantages of the comet assay, it has been applied in various types of studies, including genotoxicity studies, DNA repair studies, supplementation trials and environmental biomonitoring. The comet assay is a valuable tool in nutraceutical research, as exemplified by studies on dietary antioxidants.

APPLICATIONS OF COMET ASSAY IN DNA REPAIR STUDIES

In addition to assessing DNA damage per second, the comet assay can be used in different ways to monitor the repair of DNA damage.

6.1. Cellular repair

Cells are treated with a DNA damaging agent, and incubated in culture medium at 37˚C. At intervals, samples are taken for analysis with the comet assay. The rate at which the damage is removed indicates the efficiency of repair. Single strand breaks (induced e.g. by ionizing radiation or H2O2) are rejoined by most cell types quite rapidly, typically within 10-30 min. Oxidized bases (detected with the use of FPG or Endonuclease III) are repaired by base excision repair with at 0.5 of a few hours (Collins and Horvathova, 2001). UV-induced lesions are repaired by nucleotide excision repair with a similar time course. Even after embedding in agarose, cells are capable of repair (Collins and Horváthová, 2001). Slides bearing gels can simply be immersed in culture medium at 37˚C. A very sensitive method for following nucleotide excision repair depends on the fact that the repair synthesis stage can be inhibited with DNA polymerase inhibitors, aphidicolin or cytosine arabinoside. This leads to the accumulation of repair intermediates – normally transient strand breaks. Very low doses of UV-induced damage can be detected in this way (Gedik *et al.,* 1992).

6.2. In vitro repair

An alternative approach was devised for use in human biomonitoring studies, to assess differences between individuals in the capacity for DNA repair. An extract is prepared from lymphocytes, and incubated with a standard substrate of cells, embedded in agarose and lysed as in the standard assay. These substrate cells have previously been treated with a photosen sitizer plus visible light to induce oxidative damage in the DNA, and the effect of the extract on DNA repair rates is determined. The method has demonstrated a stimulation of repair by kiwifruit supplementation (Collins *et al.,* 2003) and has been used in a study of workers in asbestos and mineral fiber factories to investigate the range of inter-individual variation (Dušinská *et al.,* 2004). The method could be adapted to look at other repair pathways, and is suitable to investigate the effect of nutraceutical on DNA repair.

Table 2: Overview of some human supplementation studies that have used the comet assay to assess effects on DNA damage (Bajpayee *et al.*, 2005).

Treatment	Subjects	Study design	Measure	Result/Conclusion	Reference
3 treatments: vitamin C 1g; α-tocopherol 1g; β-carotene 45mg	Healthy, non-fasting subjects; 6 smokers 6 non-smokers	Single dose acute response at up to 24h post- supplementation	Damage after H_2O_2 treatment, 72 & 240μM	Vitamin C: both groups showed greater resistance against H_2O_2 –induced damage at 2-4h post ingestion. For vitamin E and β-carotene: both groups showed greater resistance against H_2O_2- induced damage at 18-24h post ingestion. DNA in cells from smokers was more sensitive to H_2O_2	Panayiotidis & Collins, 1997
4 treatments: Breakfast + 500mg vitamin C ; Breakfast + 500mg vitamin C; Breakfast; 500mg vitamin C	Healthy subjects n=6 n=3 n=2 n=2	Acute response study: samples collected at: 0,1 h 0,1,2,4,6 h 0,0.5, 1,2 h 0,0.5, 1,2 h	Damage after ionizing radiation, 0.5,1,2 Gy	Increased resistance after ascorbic acid; maximum protection at 4h	Green et al., 1994
3 treatments: β-carotene 15 mg/day; Lutein 15 mg/day; Lycopene 15 mg/day	Healthy subjects n=8	1 week supplementation, 3 weeks' washout period between treatments	Cells treated with H_2O_2, 100μM; damage assessed after 0, 2,4,8,24 h incubation of cells in complete medium, 37°C to assess recovery	Recovery from damage faster after β-carotene supplementation; enhanced recovery also after lycopene, but only in those subjects whose plasma lycopene level increased; no enhancement of repair by lutein, even though plasma levels increased 2- fold	Torbergsen & Collins, 2000
3 treatments: 500mg vitamin C; 400 IU vitamin E; 500mg vitamin C + 400 IU vitamin E	Healthy, fasting subjects n=12	Placebo-controlled, double-blinded, multiple cross-over trial; acute response; samples collected at 0, 90min, 180 min and 24h post-ingestion	Damage after H_2O_2 tretatment, 240μM and basal strand breaks (no enzyme treatment)	No evidence of protection or damage by vitamin C, vitamin E or both.	Choi et al., 2004
2 treatments: 200g fried onions; 200g fried onions + 100g fresh tomatoes	Healthy women n=6	Acute response, cross-over trial; samples collected at 0,4,8,and 24h.	Damage after H_2O_2, 50&200μM, and basal damage as Endo III sensitive sites	More resistance to H_2O_2 at 4 & 8h after onions, but not after onions+tomatoes; lower basal damage at 8 & 24h after onions, and after 4 & 8 h after onions+tomatoes	Boyle et al., 2000
1 treatment: 500ml homogenized kiwifruit (no skin; equivalent to ~8 fruits)	Healthy subjects n=6	Controlled, cross-over, single dose, acute response study. Sample collected at 0,1,3,8 & 24h post ingestion	Damage after treatment with H_2O_2, 1mM, and basal damage as Endo III & FPG sensitive sites	Increased resistance to H_2O_2 at 8 & 24 h; no effect on basal damage.	Collins et al., 2001a
Combined treatment: vitamin C 100mg + vitamin E 280mg + β-carotene 25mg per day	50 smokers 50 non-smokers	Placebo controlled double blinded study; 20 weeks supplementation	Damage after treatment with H_2O_2, 30,100, 300μM, and basal damage as Endo III sensitive sites	Supplementation caused increased resistance against 100 & 300 μM H_2O_2in both groups; decreased basal damage also seen, with greater effect in smokers; this group had higher basal damage at entry.	Duthie et al., 1996
2 treatments: cooked carrots 200g; α-carotene 3.7mg/day + β-carotene 8.2 mg/day (same as in carrots)	Men, non-smokers; n=11 n=11	Placebo controlled parallel study, 3 weeks supplementation	Cells treated with H_2O_2 100μM, single strand damage (no enzymes) assessed after 0, 2, 3, 4h recovery; basal strand breaks (no enzymes) also measured	Enhancement of recovery after carotene supplement, but not after cooked carrots; no effect on strand breaks	Astley et al., 2004

COMET ASSAY IN LOWER ANIMAL MODEL (INVERTEBRATE)

Tetrahymena thermophila is a unicellular protozoan widely used for genetic studies due to its well characterized genome. Its uniqueness lies in the fact that it has a somatic and a germ nucleus in the same cell. Therefore, it has been validated as a model organism for assessing DNA damage using a modified Comet assay protocol standardized with known mutagens such as phenol, hydrogen peroxide, and formaldehyde (Lah *et al.*, 2004). The method was then used for the assessment of genotoxic potential of influent and effluent water samples from a local municipal wastewater treatment plant (Lah *et al.*, 2004). The method provided an excellent, low-level detection of genotoxicants and proved to be a cost effective and reliable tool for genotoxicity screening of wastewater.

Studies have been carried out on various aquatic (marine and freshwater) and terrestrial invertebrates (Table 3). The genotoxicity assessment in marine and freshwater invertebrates using the assay has been reviewed (Cotelle and Ferard, 1999; Lee and Steiner, 2003). Cells from hemolymph, embryos, gills, digestive glands, and coelomocytes from mussels, zebra mussel *(Dreissena polymorpha),* clams *(Mya arenaria),* and polychaetes *(Nereis virens)* have been used for ecogenotoxicity studies using the Comet assay. DNA damage has also been assessed in earthworms and fruit fly, Drosophila, (Bilbao *et al.*, 2002; Mukhopadhyay *et al.*, 2003). The Comet assay has been employed to assess the extent of DNA damage at polluted sites in comparison to reference sites in the environment, and in the laboratory, it has been widely used as a mechanistic tool to determine pollutant effects and mechanisms of DNA damage.

COMET ASSAY IN MUSSELS

Freshwater and marine mussels have been used to study the adverse effect of contaminants in the aquatic environment, as they are important pollution indicator organisms. These sentinel species are adversely affected by pollution in the water bodies and thus provide the potential for environmental biomonitoring. The Comet assay in mussels can be used to detect a reduction in water quality caused by chemical pollution (Frenzilli *et al.*, 2001; Jha *et al.*, 2005). *Mytilus*

edulis has been widely used for Comet assay studies to evaluate DNA strand breaks in gill and digestive gland nuclei due to polycyclic aromatic hydrocarbons (PAHs) including benzo[a]pyrene (B[a]P) and oil spills with petroleum hydrocarbons. However, the damage returned to normal levels after continued exposure to high dose (20 ppb-exposed diet) of B[a] P for 14 days. This was attributed to an adaptive response in mussels to prevent the adverse effects of DNA damage. The green-lipped mussels *(Perna viridis)* also showed a similar result to B[a] P in water. Significant levels of inter individual variability, including seasonal variations in DNA damage, have been reported from some studies in both laboratory and field (Frenzilli *et al.,* 2001). Baseline monitoring has thus to be carried out over long time intervals. Temperature dependent DNA damage was observed in hemocytes of freshwater mussel *Dreissena polymorpha* (Buschini *et al.,* 2003), showing that the mussels are sensitive towards change in water temperatures. Thus, monitoring ecogenotoxicity with these species should take into account variations in temperatures. Findings have also suggested that antioxidant supplementation can improve the sensitivity of the Comet assay by lowering the baseline damage in untreated animals. Villela used the golden mussel *(Limnoperna fortunei)* as a potential indicator organism for freshwater ecosystems due to its sensitivity to water contaminants. Comet assay in hemocytes of freshwater Zebra mussel, *D. polymorpha Pallas*, was used as a tool in determining the potential genotoxicity of water pollutants (Bolognesi *et al.,* 2004; Riva *et al.,* 2007).

Klobucar suggested the use of Comet assay in haemocytes from caged, non-indigenous mussels as a sensitive tool for monitoring genotoxicity of freshwater. DNA damage and repair studies in vent mussels, *Bathymodiolus azoricus,* have been carried out to study the genotoxicity of naturally contaminated deep-sea environment. The vent mussels demonstrated similar sensitivity to environmental mutagens as that of coastal mussels and thus could be used for ecogenotoxicity studies of deep sea waters using the Comet assay. In vitro Comet assay has also been used in cells of mussels. Dose–response increases in DNA strand breakages were recorded in digestive gland cells hemocytes and gill cells of *M. edulis* exposed to both direct (hydrogen peroxide and 3-chloro-4-(dichloromethyl)-5- hydroxy-2[5H]-furanone) and indirect (B[a]P, 1-nitropyrene, nitrofurantoin and N-nitrosodimethylamine) acting genotoxicants. Digestive gland cells of *Unio tumidus* were also used for in vitro studies of DNA damage and repair due to pro-oxidative effect of polyphenolic compounds. Wilson demonstrated potential application of the Comet assay to gill cells of *M. edulis* as a potential in vitro screen for agents destined for release or disposal into the marine environment.

COMET ASSAY IN BIVALVES

Coughlan *et al.,* (2002) showed that the Comet assay could be used as a tool for the detection of DNA damage in clams *(Tapes semidecussatus)* as biomonitor organisms for sediments. Significant DNA strand breaks were observed in cells isolated from haemolymph, gill, and digestive gland from clams exposed to polluted sediment (Coughlan *et al.,* 2002; Hartl *et al.,* 2004). Comet assay was used for the assessment of sperm DNA quality of cryopreserved semen in Pacific oyster *(Crassostrea gigas),* as it is widely used for artificial fertilization. Gielazyn demonstrated the use of lesion-specific DNA repair enzyme formamidopyrimidine glycosylase (Fpg) to enhance the usefulness and sensitivity of the Comet assay in studying oxidative DNA damage in isolated haemocytes from oyster *(Crassostrea virginica)* and clam *(Mercenaria mercenaria).* The studies in mussels have shown the Comet assay to be a sensitive, but nonspecific, molecular biomarker of genotoxicity. One of the drawbacks when applying single-cell gel electrophoresis to field populations may be the adaptability of the animals to high concentrations of contaminants (example, B[a] P), which may pose a major problem. Also, seasonal variation and temperature altered both DNA damage baseline levels in untreated animals and cell sensitivity towards environmental pollutants under in vitro conditions (Buschini *et al.,* 2003; Hartl *et al.,* 2004). The Comet assay detecting DNA strand breaks has demonstrated that higher basal levels of DNA damage are observed in marine invertebrates; hence, the protocol followed in these animals should be considered for biomonitoring the ecogenotoxicity of a region.

COMET ASSAY IN EARTHWORM

The Comet assay applied to earthworms is a valuable tool for monitoring and detection of genotoxic compounds in terrestrial ecosystems (Table 3). As the worms feed on the soil they live in, they are a good indicator of the genotoxic potential of the contaminants present in the soil and thus used as a sentinel species. Verschaeve demonstrated a dose–response with the extent of DNA damage in coelomic leucocytes (coelomocytes) of earthworms *(Eisenia foetida)*

from soil treated with different chemicals as an indication of soil pollution. Coelomocytes from *Eisenia foetida* demonstrated increased DNA damage when worms were exposed to soil samples from polluted coke oven sites or industrialized contaminated areas (Xiao *et al.*, 2006) and even sediment samples from polluted river system. An insecticide, parathion, produced DNA strand breaks at all-time points and doses in the sperm cells of *Eisenia foetida* (Bustos-Obregon and Goicochea, 2002), while dose–effect relationships were displayed by two pesticides, Imidacloprid and RH-5849, in the same species, showing that pesticides could also have adverse effects on non-target species. In vitro exposure of coelomocytes primary cultures to nickel chloride as well as whole animals either in spiked artificial soil water or in spiked cattle manure substrates exhibited increased DNA strand breaks due to the heavy metal. The eleocytes, a subset of coelomocytes, exhibited increased DNA strand breaks under both in vitro and in vivo conditions and could be used as a sensitive biomarker for genotoxicity in earthworms. Another earthworm, *Aporrectodea longa* (Ude), when exposed to soil samples spiked with B[a]P and/or lindane, demonstrated intestinal cells to be more sensitive to the effect of the genotoxicants than the crop/gizzard cells. Fourie and co-workers used five earthworm species *(Amynthas diffringens, Aporrectodea caliginosa, Dendrodrilus rubidus, Eisenia foetida,* and *Microchaetus benhami)* to study genotoxicity of sublethal concentrations of cadmium sulphate, with significant DNA damage being detected in *Eisenia foetida* followed by *Dendrodrilus rubidus* and *Aporrectodea caliginosa*. The study showed a difference in sensitivity of species present in an environment and its influence on the genotoxicity risk assessment. Hence, for environmental biomonitoring, specific species have to be kept in mind to reduce false negative results.

COMET ASSAY IN *DROSOPHILA MELANOGASTER* (FRUIT FLY)

The simple genetics and developmental biology of *Drosophila melanogaster* has made it the most widely used insect model and has been recommended as an alternate animal model by the European Centre for the Validation of Alternative Methods (ECVAM; Benford *et al.*, 2000). Recently, Drosophila has evolved as a model organism in toxicological studies (Mukhopadhyay *et al.*, 2003). D. melanogaster has also been used as an in vivo model for assessment of genotoxicity using Comet assay (Bilbao *et al.*, 2002; Mukhopadhyay *et al.*, 2003; Table 2). Neuroblast cells of third instar larvae, DNA repair deficient in nucleotide excision repair (mus201), and a mechanism of damage bypass (mus308) have been used for mechanistic studies (Bilbao *et al.*, 2002). Third instar larvae of D. melanogaster (Oregon R +) were validated for genotoxicity assessment using a modified Comet assay. As the cells of Drosophila are smaller than mammalian cells, modifications in the Comet assay were done, Example; higher concentration of agarose (for the smaller size of Drosophila cells), removal of dimethyl sulfoxide (DMSO) from lysing solution (DMSO is toxic to the cells), and lower electrophoresis time (for improved performance of the assay). This modified protocol was validated in gut and brain cells using well-known alkylating agents, i.e., ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), ethyl- N-nitrosourea (ENU), and cyclophosphamide (CP), which were mixed in standard Drosophila diet and produced a significant dose-dependent response. Cypermethrin, a synthetic parathyroid, even at low concentrations (at 0.002 ppm), and leachates of industrial waste produced significant dose-dependent increase in DNA damage in the brain ganglia and anterior midgut of *Drosophila melanogaster* (Mukhopadhyay *et al.*, 2003). Results from Comet assay have also shown a direct correlation between the concentrations of cisplatin adducts and DNA damage in somatic cells of *Drosophila melanogaster* (GarcíaSar *et al.*, 2008). In vitro studies using Drosophila S2 cells demonstrated that the ectopically expressed DNA glycosylases (dOgg1 and RpS3) reduced the oxidized guanosine (8-OxoG) but contributed to increased DNA degradation due to one of the constituents of the DNA repair system. The studies in Drosophila have shown it to be a good alternate to animal model for the assessment of in vivo genotoxicity of chemicals using the Comet assay.

COMET ASSAY IN OTHER INVERTEBRATES

Nereis virensa, a polychaete, plays an important role in the distribution of pollutants in sediments due to their unique property of bioturbation. These worms are similar to earthworms in soil and can be used for genotoxicity assessment of sediments. Intracoelomic injection of B[a] P was given to the worms, and Comet assay was conducted on coelomocytes (De-Boeck and Kirsch-Volders, 1997). *Nereis* species was, however, not found to be suitable for assessing PAH genotoxicity probably due to its lack of metabolic capability to convert B[a] P to its toxic metabolite (De-

Boeck and Kirsch-Volders, 1997). DNA damage was assessed in neuroblast cells of brains of first instars of grasshoppers *(Chorthippus brunneus)* exposed to various doses of zinc from a polluted site to understand the mechanism of toxicity in insects due to industrial pollutants (Augustyniak *et al.,* 2006). The estuarine grass shrimp, *Palaemonetes pugio,* exposed to coal combustion residues from coal-fired electrical generation, were studied for DNA damage using Comet assay. Chronic exposure caused DNA damage in hepatopancreas cells of adult shrimps as compared to reference shrimp. Comet assay in planarians is an important test for environmental monitoring studies, as these are simple organism with high sensitivity, low cost, and high proliferative rate. The genotoxic potential of water from Diluvio's Basin was evaluated in planarians where increase in pollutants towards the basin led to an increase in the DNA damage in these species. Significant increase of primary DNA damage was observed in planarian cells due to Norflurazon, a bleaching herbicide, and copper sulphate when compared to the control animals. These studies have also shown the use of Comet assay in biomonitoring diverse environmental conditions utilizing sentinel species.

Table 3: Comet Assay Assessment of DNA damage in lower animal models (Invertebrate)

Lower Animal Model	Agent Tested	Cell Used	Dna Damaged	References
Tetrahymena thermophila	Phenol, hydrogen peroxide, and formaldehyde, influent and effluent water samples	Whole animal in vivo	↑	Lah *et al.,* 2004
Bivalves	-Polybrominated diphenyl ethers (pbdes)		↑↑	
	-Sodium hypochlorite and chlorine dioxide) and peracetic acid		↑	Riva *et al.,* 2007
Freshwater bivalve zebra mussel *(Dreissena polymorpha)*		Hemocytes		Bolognesi *et al.,* 2004
	-Pentachlorophenol		↑	
	-Varying temperatures		↑	Buschini *et al.,* 2003
	Polluted waters		↑	
Mytilus edulis	-Cadmium (Cd) and chromium (Cr)	Gills	–	
	-Styrene	Haemolymph cells	↑	
	-Tritium	Haemocytes	↑	
	-Marine waters (Denmark), French Atlantic Coast	Gill and haemolymph	↑	Jha *et al.,* 2005
	-Polycyclic aromatic hydrocarbons	Gill and haemocytes	↑	
	-Seasonal variation	Haemocytes	↑	
Freshwater mussels *(Unio tumidus)*	Polyphenols	Digestive gland cells	↑	
Golden mussel *(Limnoperna fortunei)*	Guaíba Basin water	Haemocytes	↑	
Bivalve mollusc *Scapharca inaequivalvis*	Organotin compounds (MBTC, DBTC and TBTC)	Erythrocytes	↑	Gabbianelli *et al.,* 2006
Mytilus galloprovincialis	Environmental stress	Haemocytes	↑	
	Heavy oil spill	Gills	-	Frenzilli *et al.,* 2001
	Cadmium	Digestive gland cells	-	Dailianis *et al.,*2005
Oyster *(Crassostrea gigas)*	Cryopreservation	Spermatozoa	↑	
Manila clam *(Tapes semidecussatus)*	Sediment-bound contaminants	Haemolymph, gill and digestive gland	↑	Coughlan *et al.,* 2002; Hartl *et al.,* 2004
Earthworms *Eisenia foetida*	-Chemical-treated soil	Coelomocytes	↑Dose-dependant	
	-Soil from coke ovens	Coelomocytes	↑	
	- Soil from industrialized contaminated areas	Coelomocytes	↑	Xiao *et al.,* 2006
	- Sediment from polluted river	Coelomocytes	↑	Bustos-Obregon and Goicochea, 2002
	-Wastewater irrigated soil	Coelomocytes	↑	
	-Commercial parathion			

		Coelomocytes	↑	
Aporrectodea longa (Ude)	Soil samples spiked with benzo[a]pyrene (B[a]P) and/or lindane	Intestine and crop/gizzard cells	↑ Intestine > crop	
Other invertebrates Fruitfly *(Drosophila melanogaster)*	- Ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), Nethyl-N-nitrosourea (ENU) and cyclophosphamide (CP)	Gut and brain cells of first instar larvae	↑	Mukhopadhyay *et al.*, 2003; García*Sar et al.*, 2008
	- Cypermethrin		↑	
	- Lechates of industrial waste		↑	
	- Cisplatin		↑	

Where - ↑: significant increase in DNA damage, ↑↑: highly significant increase in DNA damage, ↓: decrease in DNA damage, –: no DNA damage reported.

COMET ASSAY IN HIGHER ANIMAL MODEL (VERTEBRATE)

Studies of vertebrate species where the Comet assay is used include fishes, amphibians, birds, and mammals. Cells (blood, gills, kidneys, and livers) of different fishes, tadpoles and adult frogs, as well as rodents have been used for assessing in vivo and in vitro genotoxicity of chemicals, and human biomonitoring has also been carried out employing the Comet assay (Table 4).

COMET ASSAY IN FISHES

Various fishes (freshwater and marine) have been used for environmental biomonitoring, as they are endemic organisms, which serve as sentinel species for a particular aquatic region, to the adverse effects of chemicals and environmental conditions. The Comet assay has found wide application as a simple and sensitive method for evaluating in vivo as well as in vitro DNA damage in different tissues (gills, liver, and blood) of fishes exposed to various xenobiotics in the aquatic environment (Table 4).

Environmental biomonitoring to assess the genotoxic potential of river waters has been carried out in hepatocytes of chub *(Leuciscus cephalus;* Winter *et al.*, 2004), erythrocytes of mullet *(Mugil sp.),* sea catfish *(Netuma sp.;* De-Andrade *et al.*, 2004a; De-Andrade *et al.*, 2004b), bullheads *(Ameiurus nebulosus),* and carps *(Cyprinus carpio;* Buschini *et al.*, 2004). Basal level of DNA damage has been shown to be influenced by various factors, such as temperature of water in erythrocytes of mullet and sea catfish (De-Andrade *et al.*, 2004a; De-Andrade *et al.*, 2004b), age and gender in dab *(Limanda limanda;* Akcha *et al.*, 2003), and exhaustive exercise in chub (Aniagu *et al.*, 2006). Therefore, these factors should be accounted for during environmental biomonitoring studies for genotoxicity. The sensitivity of the assay may be affected by high intra individual variability (Akcha *et al.*, 2003). The protocol and experimental conditions used for the Comet assay for monitoring marine ecosystems may lead to differences in the obtained results (Belpaeme *et al.*, 1998). The use of chemical and mechanical procedures to obtain cell suspension may also lead to DNA damage. Anesthesia did not contribute towards DNA damage in vivo in methyl methanesulfonate (MMS)-treated fishes, and the anesthetic benzocaine did not alter the DNA damage in erythrocytes after in vitro exposure to MMS or H2O2 (De-Miranda Cabral Gontijo *et al.*, 2003). Hence, keeping in mind animal welfare, multisampling in the same fish can be conducted. In vitro studies on fish hepatocytes, primary hepatocytes and gill cells, as well as established cell lines (with metabolic competence; Nehls and Segner, 2002) using the Comet assay have also been conducted to assess the genotoxicity of chemicals in water samples. The antioxidant potential of indolinic and quinolinic nitroxide radicals, tannins, and low concentrations (<10 μM) of diaryl tellurides and ebselen, an organoselenium compound, in oxidative DNA damage has been studied in nucleated trout *(Oncorhynchus mykiss)* erythrocytes for use of these compounds in biological systems. Kammann demonstrated the Comet assay in isolated leukocytes of carp as an in vitro model for evaluating genotoxicity of marine sediment extracts and increased sensitivity of the method with the use of DNA repair inhibitor, 1-beta-D-arabinofuranosylcytosine (ara C). Comet assay with fish cell lines may be a suitable tool for in vitro screening of environmental genotoxicity; however, the metabolizing capabilities of the cell line need to be taken into account. Cryopreservation has been shown to induce DNA strand breaks in spermatozoa of trout (Cabrita *et al.*, 2005), sea bass *(Dicentrarchus labrax;* Zilli *et al.*, 2003), and gilthead sea bream *(Sparus aurata;* Cabrita *et al.*, 2005). The DNA damage was prevented by the

addition of cryopreservants such as bovine serum albumin and dimethyl sulfoxide *(*Zilli *et al.,* 2003). These studies have demonstrated the sperm Comet assay as a useful model in determining the DNA integrity in frozen samples for commercially cultured species. These studies have demonstrated the usefulness of the Comet assay in fishes as a model for monitoring genotoxicity of aquatic habitats using these indicator animals.

COMET ASSAY IN AMPHIBIANS

Comet assay in amphibians has been carried out at adult and larval stages for eco-genotoxicity of aquatic environments, and studies until 1999 have been well reviewed by Cotelle and Ferard (1999). The animals chosen for the Comet assay act as sensitive bioindicators of aquatic and agricultural ecosystems (Table 4). The animals were either collected from the site (in situ) or exposed to chemicals under laboratory/natural conditions. Erythrocytes from tadpole of two sentinel species *Rana clamitans* and *Rana pipiens* have been used for in situ genotoxicity monitoring of water bodies. *R. clamitans* tadpoles collected from agricultural regions showed significantly higher ($P<0.001$) DNA damage than tadpoles collected from sites of little or no agriculture. Similarly, R. pipiens tadpoles collected from industrial sites showed significantly higher ($P<0.001$) DNA strand breaks than samples from the agricultural areas. The higher levels of DNA damage may be due to the pesticides used in the agricultural region. Variation in DNA damage due to sampling time and during various metamorphosis states was also observed. Hence, for biomonitoring environmental genotoxicity using the Comet assay, pooling of early tadpole phases could be helpful. Studies have also been conducted on caged tadpoles in areas where indigenous population is not present due to ecological imbalance from pollution. *Rana clamitans* and American toad *(Bufo americanus)* tadpoles were caged at polluted reference site and demonstrated significant ($P<0.05$) increases in DNA damage, relative to control tadpoles in the laboratory. These results demonstrated that caged tadpoles could be used for monitoring genotoxicity of water habitats that do not support the survival of tadpoles, Example, large lakes and aquatic areas near high industrial activity. Huang have shown the genotoxicity of petrochemicals in liver and erythrocytes of toad *Bufo raddeis.* DNA damage was found to be positively correlated to the concentration of petrochemicals in liver, pointing to the fact that liver is the site for metabolism and may be a good marker for studying genotoxicity of compounds which require metabolic activation. Effect of polyploidy on bleomycin-induced DNA damage and repair in *Xenopus laevis* (pseudotetraploid) and *Xenopus tropicalis* (diploid) was studied using Comet assay (Banner *et al.,* 2007). The *Xenopus tropicalis* was more sensitive with lower capacity for repair than *Xenopus laevis,* showing that polyploidy protects DNA damage and allows rapid repair, and hence, these species may be used as a good model for DNA damage and repair studies.

COMET ASSAY IN BIRD

There are few studies involving Comet assay in birds (Table 4). Genetic damage due to a mining accident involving heavy metals has been reported in free-living, nestling white storks *(Ciconia ciconia)* and black kites *(Milvus migrans)* from southwestern Spain (Baos *et al.,* 2006); however, species specific and intra species differences were observed. Faullimel *et al.,* (2005) showed that the neutral Comet assay could be used to study the impact of freezing and thawing on DNA integrity in breast fillets and liver cells of frozen chicken. Frankic reported that T-2 toxin and deoxynivalenol (DON) induced DNA fragmentation in chicken spleen leukocytes which was abrograted by dietary nucleotides. Kotłowska have demonstrated increased DNA fragmentation in turkey sperm after 48 h of liquid storage and might be helpful in evaluating the DNA integrity for artificial insemination.

COMET ASSAY IN RODENT

Mice and rats have been widely used as animal models for the assessment of in vivo genotoxicity of chemicals using the Comet assay (Table 4). The in vivo Comet assay has been accepted by the UK Committee on Mutagenicity testing of chemicals in food, consumer products, and environment (COM 2000) as a test for assessing DNA damage and is recommended for follow-up testing of positive in vitro findings. A positive result in the in vivo Comet assay assumes significance if mutagenic potential of a chemical has already been demonstrated in vitro. Within a battery of tests, Comet assay finds place as a supplemental in vivo test which has been accepted by international guidelines (Brendler Schwaab *et al.,* 2005). There are specific guidelines for the performance of Comet assay in vivo for reliable results (Tice *et al.,* 1995; Hartmann *et al.,* 2003; Burlinson *et al.,* 2007). Multiple organs of mouse/rat including brain, blood, kidney, lungs liver, and bone marrow have been utilized for the

comprehensive understanding of the systemic genotoxicity of chemicals (Sasaki *et al.*, 2000). The most important advantage of the use of Comet assay is that DNA damage in any organ can be evaluated without the need for mitotic activity and DNA damage in target as well as non-target organs can also be seen (Sasaki *et al.*, 2000). A comprehensive data on chemicals representing different classes, example, PAHs, alkylating compounds, nitroso compounds, food additives, etc., that caused DNA strand breaks in various organs of mice were compiled by Sasaki *et al.*, (2000). The mouse or rat organs exhibiting increased levels of DNA damage were not necessarily the target organs for carcinogenicity. Therefore, for the prediction of carcinogenicity of a chemical, organ-specific genotoxicity was necessary but not sufficient (Sasaki *et al.*, 2000). The Comet assay can be used as an in vivo test apart from the cytogenetic assays in hematopoietic cells and also for those compounds which have poor systemic bioavailability. Different routes of exposure in rodents have been used, e.g., intra peritoneal (Ansari *et al.*, 2004), oral (Ansari *et al.*, 2005), and inhalation (Valverde *et al.*, 2002), to study the genotoxicity of different chemicals.

The route of exposure is an important determinant of the genotoxicity of a chemical due to its mode of action. The in vivo comet assay helps in hazard identification and assessment of dose–response relationship as well as mechanistic understanding of a substances mode of action. Besides being used for testing the genotoxicity of chemicals in laboratory-reared animals, Comet assay in wild mice can be used as a valuable test in pollution monitoring and environmental conservation. In vivo Comet assay in rodents is an important test model for genotoxicity studies, as many rodent carcinogens are also human carcinogens, and hence, this model not only provides an insight into the genotoxicity of human carcinogens but also is suited for studying their underlying mechanisms.

COMET ASSAY IN HUMAN

Comet assay is a valuable method for detection of occupational and environmental exposures to genotoxicants in humans and can be used as a tool in risk assessment for hazard characterization (Albertini *et al.*, 2000; Dusinska *et al.*, 2004; Table 3). DNA damage assessed by the Comet assay gives an indication of recent exposure and at an early stage where it could also undergo repair, and thus, it provides an opportunity for intervention strategies to be implemented timely. The assay can be conducted in the same population after removal of genotoxicant/dietary intervention to detect the extent of reduction in DNA damage. The assay is a noninvasive technique compared to other DNA damage techniques (chromosomal aberrations, micronucleus), which require larger sample (~2–3 ml) as well as proliferating cell population (or cell culture). Human biomonitoring using the Comet assay is advantageous, as it is rapid, cost-effective, easy compilation of data and concordance with cytogenetic assays (Faust *et al.*, 2004). The assay has been widely used in studying DNA damage and repair in healthy individuals (Bajpayee *et al.*, 2002; Bajpayee *et al.*, 2005; Betti *et al.*, 1995; Collins, 2004) in clinical studies (Corrie *et al.*, 2005) as well as in dietary intervention studies and in monitoring the risk of DNA damage resulting from occupational (Srám and Binková, 2000), environmental, oxidative DNA damage (Cavallo *et al.*, 2006b), exposures or lifestyle (Avogbe *et al.*, 2005). White blood cells or lymphocytes are the most frequently used cell type for Comet assay in human biomonitoring studies (reviewed by Angerer *et al.*, 2007; Faust *et al.*, 2004); however, other cells have also been used, Example, buccal cells (Szeto *et al.*, 2003), nasal, sperm (Delbes *et al.*, 2007; Singh *et al.*, 2003), epithelial (Graham-Evans *et al.*, 2004), and placental cells (Augustowska *et al.*, 2007). The Comet assay has been used as a test to predict the risk for development of diseases (renal cell carcinoma, cancers of the bladder, oesophagus, and lung) due to susceptibility of the individual to DNA damage. The in vitro Comet assay is proposed as an alternative to cytogenetic assays in early genotoxicity/photogenotoxicity screening of drug candidates as well for neurotoxicity. Certain factors like age, diet, lifestyle (alcohol and smoking), as well as diseases have been shown to influence the Comet assay parameters, and for interpretation of responses, these factors need to be accounted for during monitoring human genotoxicity (Anderson *et al.*, 2001). Human biomonitoring studies using the Comet assay provide an efficient tool for measuring human exposure to genotoxicants, thus helping in risk assessment and hazard identification.

Table 4: Comet Assay Assessment of DNA damage in higher animal models (Vertebrate)

Higher Animal Model	Agent Tested	Cell Used	Dna Damaged	References
		VERTEBRATES		
FISHES				
Chub *(Leuciscus cephalus)*	PAHs, PCBs, organochlorine pesticides (OCPs), as well as heavy metals	Hepatocytes	↑	Winter *et al.*, 2004
Freshwater teleost fish *(Mystus vittatus)*	Exhaustive exercise	Erythrocytes	↑ In all cells	Aniagu *et al.*, 2006
Freshwater goldfish *(Carassius Auratus)*	Endosulfan	Gill, kidney, and erythrocytes	↑↑ Dose-dependent	Cavas and Könen, 2007
Zebrafish *(Danio rerio)*	Technical herbicide Roundup containing Glyphosate salt ADDB and PBTA-6 Surface waters of German rivers, Rhine and Elbe	Erythrocytes, Hepatocytes and gill cells	↑	
AMPHIBIANS				
Amphibian larvae *(Xenopus laevis* and *Pleurodeles waltl)*	Cadmium (CdCl2) Captan (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide)	Erythrocytes	↑ Concentration-and time dependent	Mouchet *et al.*, 2007
Amphibian larva *(Xenopus laevis)*	Benzo(a)pyrene, ethyl methanesulfonate	Erythrocytes	↑	Mouchet *et al.*, 2007
Toad *(Bufo raddei)*	Petrochemical (mainly oil and phenol) polluted area	Liver cells and erythrocytes	↑	
Toad *(Xenopus laevis* and *Xenopus tropicalis)*	Bleomycin induced DNA damage and repair	Splenic lymphocytes	↑ DNA damage X. tropicalis > X. laevis DNA repair in X. laevis > X. tropicalis	Banner *et al.*, 2007
BIRDS				
Wild nestling white storks (Ciconia ciconia)	Heavy metals and arsenic	Blood cells	↑ Correlated with arsenic ↑↑	
Black kites (Milvus migrans)	Toxic acid mining waste rich in heavy metals Heavy metals and arsenic	Blood cells	↑ Correlated with copper, and cadmium ↑ (2- to 10-fold)	
Turkey	Toxic acid mining waste rich in heavy metals			Baos *et al.*, 2006 Faullimel *et al.*, 2005
Chicken	Short term storage	Sperm	↑	
Chicken	T-2 toxin and deoxynivalenol (DON) Storage conditions (4°C)	Spleen leukocytes Liver and breast muscle cells	↑ ↑ Liver cells > breast muscle cells	
RODENTS				
Aldh2 knockout mice	Ethanol	Hepatic cells	↑ Oxidative damage	
P53(+/−) mice	Melphalan	Liver, bone marrow, peripheral blood and the distal intestine	DNA cross-links in all cells tested	Cordelli *et al.*, 2004; Cordelli *et al.*, 2007
SKH-1 mice	UV A + Fluoroquinolones (clinafloxacin, lomefloxacin, ciprofloxacin) UVA + 8-methoxypsoralene (8-MOP)	Epidermal cells For fluoroquino-lones	↑↑ ↓ for MOP	
Dyslipidemic	Ageing Diesel exhaust particles	Aorta, liver, and lung	↑ Oxidative damage in liver; in lung or aorta	Valverde *et al.*, 2002

ApoE(−/−) mice	Trypanosoma cruzi infection	Peripheral blood, liver, heart, and spleen cells	↑ In heart and spleen	Ansari *et al.*, 2004; Ansari *et al.*, 2005
Balb/c mice				
CD-1 mice	Lead acetate	Nasal epithelial cells, lung, whole blood, liver, kidney, bone marrow, brain and testes	↑ In all organs on prolonged exposure; in testes	
Swiss albino mice	Sanguinarine alkaloid, argemone oil	Blood, bone marrow cells and liver	↑ Dose-dependent in blood and bone marrow	
	Cypermethrin	Brain, liver, kidney, bone marrow, blood, spleen	↑	
HUMANS				
Clinical Breast cancer patients and Controls	Radiosensitivity	Peripheral blood mononuclear cells	↑	
Normal individuals	Chlorhexidine	Buccal epithelial cells and peripheral blood lymphocytes	↑	Eren *et al.*, 2002
Ataxia telangiectasia heterozygote	X-irradiation	Peripheral	leukocytes ↑ (three times high) in patients	
Cancer (testicular cancer, lymphoma and leukemia)patients	DNA integrity	Spermatozoa	Decreased DNA integrity	
Smokers	Vitamin C	Blood lymphocyte	↓	
Technical anesthesiology staff	supplementation Vitamin E and vitamin C	Blood lymphocyte	↓ In oxidative damage	
Airport personnel	Jet fuel vapors, jet fuel combustion products	Exfoliated buccal cells and lymphocytes	↑	Cavallo *et al.*, 2006a
Agricultural workers	Pesticides	Lymphocytes	–	
Human lymphocytes	Heterocyclic amine and prevention by monomeric and dimeric flavanols and black tea Polyphenols	Lymphocyte	↓ In oxidative damage	Bakare *et al.*, 2007
	C60 Fullerenes	Lymphocyte	↑	
	Municipal sludge leachates	Lymphocyte	↑	

Where - ↑: significant increase in DNA damage, ↑↑: highly significant increase in DNA damage, ↓: decrease in DNA damage, –: no DNA damage reported.

CONCLUSION AND RECOMMENDATION

There is no much work done in the study of comet assay as a tool for detecting exposure, and its validation status as a biomarker in biomonitoring performance of cohort studies in Nigeria. However, many laboratories have carried out their own validation studies for DNA damage to optimize their research work. Moller has critically evaluated to published Comet assay data on human biomonitoring studies using blood cells from 22 countries and has established reference values for DNA damage. The large number of biomonitoring studies has indicated that the Comet assay is a useful tool for detecting exposure, and its validation status as a biomarker in biomonitoring dependent on its performance in cohort studies. The Comet assay is now well established now, and its versatility has imparted a sensitive tool to the toxicologists for assessing DNA damage. This has been demonstrated with its wide applications in assessing genotoxicity in plant and animal models, both aquatic as well as terrestrial, in a variety of organisms, tissues, and cell types. In vitro, in vivo, in situ, and biomonitoring studies using the comet assay have proven it to be a *Rossetta Stone* in the garden of genetic toxicology.

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